This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2005)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2005 Term

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Peroxynitrite: An Overview

by

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For 77:222, Spring 2005

9. February 2005

Abbreviations

3NT	3-nitrotyrosine
CO_2	Carbon dioxide
DCFH (DCDHF)	Dichlorodihydrofluorescein
DHR	Dihydrorhodamine
DCF	Dichlorofluorescein
GC	Gas chromatography
$HO^{\bullet}$	Hydroxyl radical
HPLC	High-performance liquid chromatography
$NO^{\bullet}$	Nitric oxide
$O_2^{\bullet -}$	Superoxide
$ONOO^-$	Peroxynitrite; oxoperoxonitrate (1-); nitrosodioxidanide
ONOON	Peroxynitrous acid
ONOOH*	Peroxynitrous acid, an activated form
ROS	Reactive oxygen species
RSH	Sulfhydryl
RSSR	Disulfide
SOD	Superoxide dismutase

Table of Contents

Abstract

Peroxynitrite is a strong oxidant that can be produced *in vivo* as a result of the reaction between superoxide and nitric oxide. In normal conditions, superoxide and nitric oxide free radicals react slowly. However during oxidative stress and inflammation, they create peroxynitrite anion. For obtaining peroxynitrite *in vitro*, reaction between hydrogen peroxide and nitrous acid is used. Despite its short half-life, peroxynitrite can be detected using biological oxidants or specific markers. Decomposing of peroxynitrite is pH-dependent; its level of stability and chemical reactivity depend on the isomeric form and conditions of the reaction. If present in the reasonable amounts, peroxynitrite is beneficial to the organism; however if present in excess, it can cause damage through oxidation.

Introduction

Prior to 1960, the scientific community considered free radicals to be excessively reactive for participating in regular biochemical processes. This generally held belief came under challenge when discoveries of superoxide dismutase (SOD) [1], nitric oxide, and other species took place. In the coming decades, studies demonstrated that the early belief was erroneous. It has been shown that free radicals can be produced in biochemical reactions and cause damage [2,3,4,5,6].

After establishing that free radicals could be responsible for oxidative damage, scientists commenced an intensive search for the free radicals most likely to be involved. At one time, reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), were thought to cause most of the oxidative damage *in vivo*. However, they are not reactive enough for this role. Another hypothesized radical, formed in the presence of $O_2^{\bullet -}$, was an extremely reactive hydroxyl radical ($HO^{\bullet}$). While $HO^{\bullet}$ was shown to cause significant damage *in vivo*, Beckman conclusively demonstrated that such damage only occurred when free metals and H_2O_2 were in concentrations significantly higher than what is normally found *in vivo* [7]. It was then suggested, and later shown, that the formation of peroxynitrite anion $ONOO^-$ *in vivo* [3] plays a toxic role in the organism [8]. In fact, peroxynitrite production has a greater influence in causing oxidative stress *in vivo* than $HO^{\bullet}$ production [9]. This paper will allow to become familiarized with formation and synthesis of peroxynitrite, its detection, decomposition, isomeric forms, and chemical interactions.

Formation

Peroxynitrite (also oxoperoxonitrate (1-) or nitrosodioxidanide) is an inorganic molecule of biological interest. It is a strong toxic oxidant formed from the reaction of $O_2^{\bullet -}$ and nitric oxide ($NO^{\bullet}$). In normal conditions, these two radicals are formed at different rates in the same cellular or extracellular compartment [2], which prevents massive peroxynitrite

formation. Under conditions of oxidative stress and inflammation [2,3], peroxynitrite is formed in an extremely rapid reaction close to a diffusion limit (k = 4-7 x 10^9 M^{-1} s^{-1}) [10]:

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

Peroxynitrite is not a free radical because a new N–O bond combines the unpaired electrons from nitric oxide and superoxide. In this reaction, $NO^{\bullet}$ is more "persistent" in making peroxynitrite. It is hydrophobic and can easily diffuse through membranes of several cell diameters, which increases the risk of creating peroxynitrite [11]. Under normal conditions, $NO^{\bullet}$ is degraded to nitrate through reaction with O_2; this degradation occurs in the presence of myoglobin and hemoglobin, in muscles and red blood cells respectively [6]. When $NO^{\bullet}$ ends up reacting with $O_2^{\bullet-}$, it has to compete with superoxide dismutase (SOD) that also reacts with $O_2^{\bullet-}$. It was determined that the rate of reaction that involves $NO^{\bullet}$ is up to three times faster than reaction with involvement of SOD [12]. Therefore, to prevent peroxynitrite formation, the concentrations of $NO^{\bullet}$ are kept low *in vivo*.

Superoxide, in its turn, increases risk of making peroxynitrite because it does not get degraded as quickly as $NO^{\bullet}$. In order to lower the chances of peroxynitrite production, $O_2^{\bullet-}$ does not diffuse as easily as $NO^{\bullet}$.

Synthesis *in vitro*

In order to synthesize peroxynitrite *in vitro*, the most common method is the reaction between H_2O_2 and nitrous acid (HONO) (pH~1) where peroxynitrous acid is produced:

$$H_2O_2 + HONO \rightarrow HOONO + H_2O$$

Due to the instability of peroxynitrous acid (its half-life is under 1 s), the reaction should be followed by immediate adding of an alkaline solution (0 ºC), e.g., sodium hydroxide (NaOH) [13]. The reason for using an alkaline solution is to stabilize the product as peroxynitrite [14]:

$$ONOON + HO^- \rightarrow ONOO^- + H_2O$$

The solutions of peroxynitrite can be kept at –20 ºC, where a decomposition occurs gradually with a half-life of one to two weeks [15]. In order to receive a high yield of peroxynitrite, minimal concentrations, and in ideal case no HONO, should remain in the reaction mixture [14].

Methods of Detection

In vivo detection of peroxynitrite was considered when SOD increased the lifetime of $NO^{\bullet}$ in biological systems [16]. Later peroxynitrite formation was shown in enzymatically activated macrophages [3]. It is now known that peroxynitrite forms during human pathologies. Perhaps the strongest evidence of peroxynitrite involvement is the presence of its marker, 3-nitrotyrosine (3NT), found in human tissue samples (e.g., [8]). This marker is a strong indicator of peroxynitrite presence if there are no other avenues of tyrosine nitration. The methods used for 3NT detection form two basic categories: molecular analysis using 3NT antibody-staining techniques [17] and chemical analysis using high-performance liquid chromatography (HPLC) and gas chromatography (GC) [18] with various detection techniques (i.e., electrochemical, ultraviolet detection).

In vitro detection of peroxynitrite can be performed through oxidating dihydrorhodamine (DHR) and dichlorodihydrofluorescein (DCFH). After exposing DHR and DCFH to a number of biological oxidants (visible spectra 500nm absorbance for real time monitoring), it was determined that distinctive oxidation occurred only in peroxynitrite, H_2O_2 with horseradish peroxidase, and by hypochlorous acid. If the latter production does not occur and horseradish peroxidase is absent, DHR or DCFH oxidation can be used for detecting peroxynitrite [19]. **Fig. 1** shows that increased concentrations of peroxynitrite raise the levels of oxidation products – dichlorofluorescein (DCF) and rhodamine. Three sequential additions of 4 μM peroxynitrite to 100 μM solutions of DCFH resulted in a 38.4% oxidative efficiency (DCF formation) of peroxynitrite. Addition of 4 μM peroxynitrite to 100 μM

solutions of DHR resulted in formation of a 43.6% oxidative efficiency (rhodamine formation) [19].

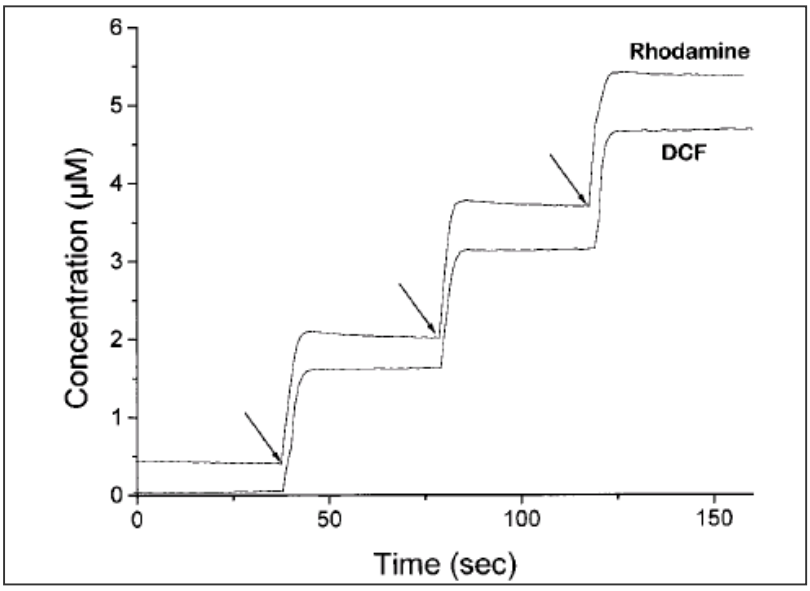

Fig. 1. Oxidation of DHR and DCFH by peroxynitrite. Arrows indicate three sequential additions of 4 μM peroxynitrite made to 100 μM solutions of either DHR or DCFH and 0.1 mM DTPA in 100 potassium phosphate, pH 7.4. Oxidative efficiency for peroxynitrite was determined by averaging the yields of rhodamine and DCF relative to the amount of peroxynitrite added [19].

Decomposition

Peroxynitrite is relatively stable in alkaline conditions. Its decomposition to nitrite and oxygen occurs slowly [20]:

$$ONOO^- \rightarrow NO_2^- + \tfrac{1}{2}O_2$$

On the contrary, at a neutral pH, decomposition of peroxynitrite is rapid (half-life is less than a second) and leads to forming peroxynitrous acid (ONOOH) (pK_a=6.75, T = 37 ºC). The acid is unstable and decays to nitrate (first order reaction) [5]:

$$ONOO^- \underset{pK_a=6.75}{\leftrightarrow} ONOOH \underset{k_1 = 4.5\ s^{-1}}{\leftrightarrow} NO_3^- + H^+$$

The first-order rate constant (k_1) for peroxynitrous acid decay is 4.5 s^{-1} (T = 37 ºC), its half-life is 0.15 s [5]. Thus, the decomposition rate of peroxynitrite is still first order and is pH dependent.

Peroxynitrite and peroxynitrous acid (ONOOH) are both reactive and damaging species. A metastable form of ONOOH, ONOOH*, is an intermediate formed during the decay of ONOOH to $NO_2^{\bullet}$. Depending on the conditions of the reaction, $ONOO^-$ can dissociate to different products. Kissner *et al*. [4] used laser flash photolysis in order to study

the behavior of peroxynitrite. The authors concluded that peroxynitrite during photolysis dissociates into $O_2^{\bullet-}$ and $NO_2^{\bullet}$:

$$ONOO^- \xrightarrow{h\nu} NO_2^{\bullet} + O_2^{\bullet-}$$

.Summary of peroxynitrite decomposition is shown in **Fig. 2**.

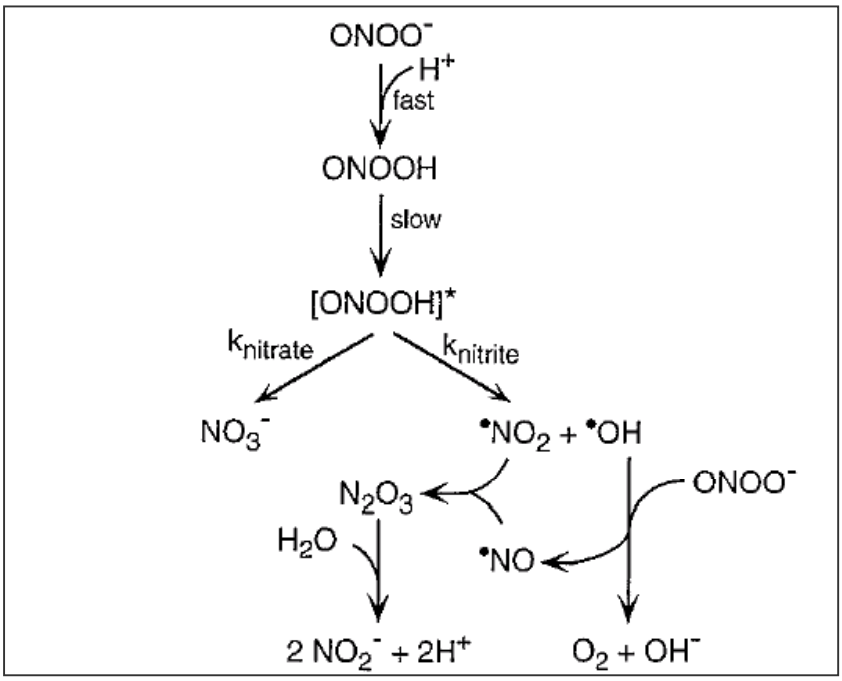

Fig. 2. Hypothetical mechanism of peroxynitrite decomposition [21].

Isomerization

Peroxynitrite exists in both *cis* and *trans* isomeric forms. The *cis* form is the most stable, and the reactivity appears to be higher in the *trans* configuration [22]. Isomeric forms are shown in **Fig. 3**:

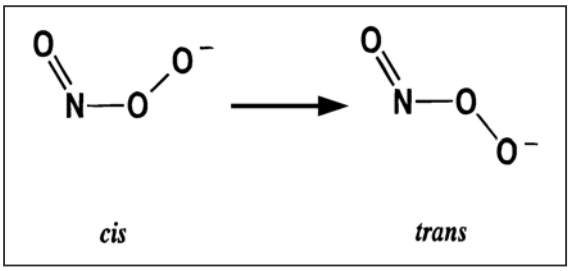

Fig. 3. Peroxynitrite isomerization [4].

Isomerization takes place during laser flash photolysis and is involved in clarifying the nature of ONOOH*. The *cis* and *trans* forms of peroxynitrite occur due to the N– O partial double bond. It is thought that a more stable *cis* conformation becomes protonated

which allows isomerization of ONOOH to *trans* form. When ONOOH decays to nitrate, perhaps then it may form ONOOH* [9].

Chemical Interactions

Carbon Dioxide Reaction. One of the important routes for peroxynitrite is reacting with carbon dioxide [23]. The reaction takes place in biological environments when carbon dioxide is in abundance. In the reaction, peroxynitrite and carbon dioxide make the nitrosoperoxycarbonate anion ($ONOOCO_2^-$) which rearranges to the nitrocarbonate anion ($O_2NOCO_2^-$) [23]:

$$\underset{\text{nitrosoperoxycarbonate}}{ONOO^- + CO_2} \rightarrow \underset{\text{nitrocarbonate}}{ONOOCO_2^-} \rightarrow O_2NOCO_2^-$$

The nitrocarbonate anion is a proximal oxidant in the reactions that are mediated with peroxynitrite [23]. Through hydrolysis, nitrocarbonate oxidizes and nitrates substrates:

$$O_2NOCO_2^- + H_2O \rightarrow CO_3^{2-} + NO_3^- + 2H^+$$

It has been demonstrated that carbon dioxide enhances tyrosine nitration by peroxynitrite [23]. Thus, carbon dioxide concentration is of high importance for peroxynitrite-mediated reactions.

Sulfhydryls. Peroxynitrite reacts directly with sulfhydryls (RSH) and yields the corresponding disulfide (RSSR) [24]:

$$ONOO^- + 2RSH \rightarrow RSSR + H_2O + NO_2^-$$

Peroxynitrite can oxidize sulfhydryl groups 10^3 times faster than H_2O_2 [25].

Nitration. Peroxynitrite will nitrate aromatics (R-Ar) to cause the corresponding nitroderivative in a reaction that is catalyzed by transition metals (M^{n+}) [24]:

$$ONOO^- + R\text{-}Ar/\ M^{n+} + 2H^+ \rightarrow R\text{-}Ar\text{-}NO_2 + M^{(n-1)} + H_2O$$

Luminol Chemiluminescence. Luminol is an almost white to yellow crystalline compound $C_8H_7N_3O_2$ that gives a brilliant bluish luminescence when treated properly [26]. For yielding light, luminol has to form an unstable endoperoxide. The decomposition of endoperoxide yields and excited state of 3-aminophthalic acid. The excited state relaxes to

the ground state by emitting a photon. For most reactions, superoxide is the intermediate of chemiluminescence. Therefore, reaction is SOD-inhibitable. Peroxynitrite was shown to be capable of inducing the reaction of chemiluminescence [27]:

$$ONOO^- + LH^- \rightarrow NO_2^\bullet + L^{\bullet -} + OH^-$$

If there is another luminol radical close to $NO_2^\bullet$, a diazaquinone (L) will be formed:

$$ONOO^- + LH^- \rightarrow NO_2^- + OH^- + L$$

This reaction has been widely used for detection of ROS. It is observed when macrophages and neutrophils go through the respiratory burst [27].

Conclusion

The reactive chemistry of peroxynitrite is such that it can be considered beneficial to the organism: it is cytotoxic to bacteria and other invading organisms [4]. However, in excessive amounts peroxynitrite can cause damage in healthy tissues. The negative effect can be prevented (i.e., by blocking excessive amounts of superoxide and nitric oxide radical), intercepted (i.e., by adding carbon dioxide) or repaired (i.e., DNA repair systems). Organism defense against excessive peroxynitrite occurs at various levels. Thus the pathways of protection against excessive peroxynitrite that are not specific to it may be also involved.

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